Molecular Testing in Solid Tumors

An Overview

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• Molecular testing in anatomic pathology is going to become more and more important during the next decade as we develop assays that can aid in diagnosis, prognosis, and predicting response to therapy. The anatomic pathologist needs to be familiar with the different assays available but also needs to be able to discern which are going to become standard of care and which will not. Three different types of tumors are reviewed: thyroid cancer, oligodendroglioma, and lung carcinoma. Molecular assays that are currently in use or on the near horizon, including translocation analyses for *RET-PTC* and *PPARγ-PAX8,* point mutation analysis for *BRAF* and epidermal growth factor receptor, and genetic loss for 1p and 19q, are discussed.

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Traditionally, pathologists have relied on the hematoxylin-eosin–stained slide to make diagnoses. Prognostic indicators were limited to those that could be seen at the light microscopic level and included such variables as surgical margin status, lymph node metastases, and perineural and angiolymphatic invasion. These tools remain at the core of pathology practice for clinical care of patients. In the past 2 decades, the analysis of protein expression by immunohistochemical staining has become an integral tool for assessment of pathology specimens. More recently, molecular testing is being integrated into very specific areas in diagnostic pathology.

Molecular testing in anatomic pathology serves various functions. The testing can be used diagnostically to assist in classifying or subclassifying tumors or other lesions. Clinical molecular testing can also be used for prognostic purposes. Finally, testing for molecular mutational events can be performed to analyze potential to respond to therapeutic agents.

The technologies that are used in assays for mutational events in anatomic pathology are often based on standard molecular biology techniques and assays. Many of the clinical assays rely on amplification of nucleic acids (polymerase chain reaction [PCR]) and hybridization techniques (fluorescent in situ hybridization [FISH]). Three types of tumors are discussed in this brief review, with particular emphasis on some of the specific molecular assays that are currently in use or in development for these organ systems. Several specific techniques are discussed in reference to clinical applicability in samples from the anatomic pathology laboratory.

THYROID CARCINOMA: *RET-PTC, PPARγ-PAX8,* AND *BRAF*

Thyroid tumors have several well-identified molecular mutations. These have given us a better understanding of pathogenesis and now are making their way into use for clinical diagnostic testing. In fact, several studies have now reported utility of molecular testing in thyroid specimens preoperatively in fine-needle aspiration samples in final pathology diagnosis.[1,2]

The 2 most common types of mutations in thyroid cancers are translocations and point mutations. Translocations, or intrachromosomal rearrangements, between the *RET* proto-oncogene and various partner genes are found in up to 40% of papillary carcinomas.[3] The most common partner genes are *H4* (*PTC1*) and *ELE1* (*PTC3*) (Figure 1). These translocations, and particularly *RET-PTC3,* are more common in radiation-induced tumors.[4]

The other translocation that is notable in thyroid cancers occurs in follicular carcinomas. Up to 40% of follicular carcinomas harbor a translocation between the *PAX8* gene and the *PPARγ* gene (*PAX8-PPARγ*).[5,6] Oncocytic carcinomas do not usually have this *PAX8-PPARγ* translocation. However, recent evidence has suggested that some cases of follicular variant of papillary carcinoma may harbor this translocation.[7]

Translocations can be difficult to detect in paraffin samples. Many translocation breakpoints occur in large introns and may not be highly clustered, making PCR from genomic DNA very difficult. The most common strategy for testing is to use the messenger RNA (mRNA), in which the introns are spliced out, resulting in consistent fusion of exons from the 2 different partner genes in the mRNA. Unfortunately, mRNA is not of high quality when obtained from paraffin samples, and so many of the translocation assays are designed for fresh or frozen tissue. Another testing option for translocations that has proven to be highly successful in paraffin-embedded tissues is FISH. Dual color probes, or chromosome-specific paints, will localize each chromosome involved in the translocation. After hybridization in the assay, the abnormal fusions or

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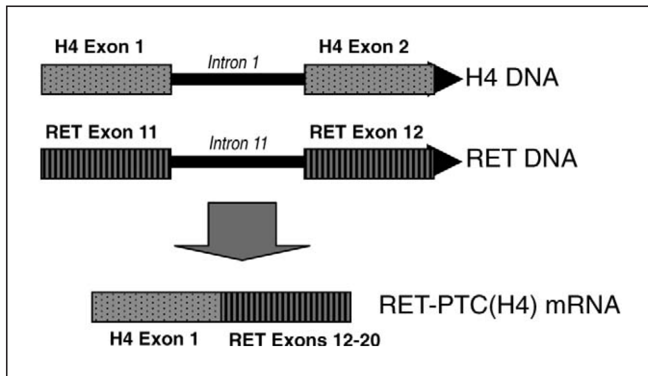

Figure 1. *This diagram demonstrates the RET-PTC1 rearrangement, in which exon 1 of the* H4 *gene is fused to exons 12 to 20 of the* RET *gene.*

breaks can be identified. Finally, the overexpression of a fusion protein driven by the translocation can be detected by immunohistochemistry in certain instances. Antibodies for RET and PPARγ have been used in this way. This approach is usually the least specific because protein expression can be increased through a variety of mechanisms.

The final notable mutation in papillary carcinoma is found in the *BRAF* gene, which encodes for a serine threonine kinase.[8,9] This point mutation is a T-to-A transition at codon 600 (nucleotide 1799). This mutation is thought to be the most common mutation in papillary carcinomas and is estimated to be found in 40% to 60% of cases.[10] The mutation is more frequently found in conventional and tall-cell variant tumors and is uncommon in follicular variant of papillary carcinoma.[5] Point mutations can be detected by a variety of assays, most of which are based in PCR techniques. These assays include standard direct gene sequencing, quantitative PCR, and newer platforms, such as pyrosequencing. All of the assays are fairly reliable for identifying cases with the characteristic *BRAF* gene mutation in papillary thyroid carcinoma.

OLIGODENDROGLIOMA AND LOSS OF 1p AND 19q

In brain tumors, concurrent loss of 1p and 19q is associated with oligodendroglioma differentiation.[11] This mutational profile has also been shown to correlate with responses to chemotherapy.[12] By contrast, loss of heterozygosity (LOH) mutations in *p53* and *p16* may be associated with a poor survival or with tumor progression.[13] Because of the strong prognostic and diagnostic value of 1p and 19q loss analysis, some institutions are testing oligodendrogliomas and other primary glial neoplasms for these genetic abnormalities.[14,15] The areas of 1p and 19q that are lost are fairly large and have been called the *minimal deletion regions.* Usually, multiple markers are used to cover the region (Figure 2).[16]

Loss of chromosomal arms can be assessed by FISH or by PCR, using an LOH analysis.[11] FISH for loss of genetic material on fixed tissue sections can be difficult to interpret because of incomplete nuclei from drop-out in tissue sectioning. However, with appropriate validation, this is an excellent diagnostic test.

Loss of heterozygosity analysis relies on our ability to discriminate between the 2 inherited copies of a particular gene. Polymerase chain reaction–based technologies have evolved to do this type of assessment using the polymorphisms in the human genome. These polymorphisms occur frequently with an estimated periodicity of at least 1 polymorphism per 1000 base pairs. Loss of heterozygosity analysis usually uses short tandem repeat polymorphisms. These are areas in the genome at which there is a short sequence of DNA that is repeated. The sequence of the repeat unit is the same from person to person, but it is repeated for a variable number of times; these polymorphisms are inherited.

For PCR assays to assess for LOH, primers are designed to flank a location-specific short tandem repeat polymorphism. In a person who is heterozygous for that gene locus, the PCR amplicons will have different lengths because there are 2 copies of the gene with different numbers of repeat units. Polymerase chain reaction products of different sizes will migrate at different speeds in both gel and capillary electrophoresis analyses. In tumor cells that have lost 1 copy of a normally heterozygous short tandem repeat polymorphism (LOH), there will be only 1 PCR product.

Both LOH and FISH assays are commonly used to detect loss of 1p and 19q in oligodendrogliomas. These assays provide valuable clinical prognostic and therapeutic information.[13]

LUNG ADENOCARCINOMA AND EPIDERMAL GROWTH FACTOR RECEPTOR

Lung carcinogenesis is complex and has predominantly been associated with smoking-related genetic damage. One of the common mutations found in lung cancer is a point mutation in the *RAS* oncogene.[17] These mutations are often clustered in 2 different hot spots and can be fairly easily detected by direct sequencing. Many other mutations in other genes, including tumor suppressor genes, have also been described in lung carcinogenesis.[18]

Figure 2. *This diagram demonstrates the approach used to select short tandem repeats covering a minimal deletion region for 1p and 19q. These short tandem repeats fall within the minimal deletion region.*

Table 1. Three Most Common Anti–Epidermal Growth Factor Receptor Drugs Currently Available and the Food and Drug Administration (FDA) Approval Status

Trade Name	Generic Name	Company	FDA Approval
Iressa	Gefinitib	AstraZeneca (Wilmington, Del)	Limited for lung
Tarceva	Erlotinib	OSI (Melville, NY); Genentech (South San Francisco, Calif)	Lung, pancreatic
Erbitux	Cetuximab	ImClone (New York, NY)	Colon

Table 2. Epidermal Growth Factor Receptor (EGFR) Sequence Mutations in Different Populations*

Clinical Feature	EGFR Mutant, %
Never-smokers	63
Smokers	22
Female	55
Male	23
Adenocarcinoma	47
Other tumor type	1.7

* These data demonstrate the percentage of cases from each clinical group of patients that have EGFR mutations from Tam et al[29] and Sugio et al[30] in studies originating in Asia.

The recent discovery of alterations in epidermal growth factor receptor (*EGFR*) in lung carcinomas has been intriguing, especially because there are drugs that are directed against *EGFR* that have become potential targeted therapies for lung carcinoma (Table 1).

Epidermal growth factor receptor is also known as c-*Erb-B1* and *HER1.* Epidermal growth factor receptor and the closely related family members *HER2/neu* (c-*Erb-B2*) and *Erb-B3* and *Erb-B4* are all cell membrane receptors that have intrinsic tyrosine kinase activities. Epidermal growth factor receptor is a 170-kd protein, 1186 amino acid transmembrane protein that has an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. As with other oncogenes, *EGFR* can be assessed at several different levels: At the genomic level, we can identify mutations in the DNA sequence or copy number changes. At the mRNA level and the protein expression level, the amount of gene expression or the protein expression can be analyzed. In lung cancers, studies have detected alterations in *EGFR* at each level.[19,20]

In lung carcinomas, overexpression of EGFR by immunohistochemistry is often seen. Variable results have been reported regarding the prognostic significance of EGFR expression. Gene amplification by FISH has also been described in lung carcinomas, and recent reports do suggest that amplification has prognostic and therapeutic implications.[21,22]

One of the most interesting *EGFR* alterations has been recently described.[23–25] Mutations have been found clustered in the tyrosine kinase domain of the *EGFR* gene, occurring almost exclusively in lung carcinomas. They are thought to be highly specific to certain populations of patients with specific tumors: young women, never-smokers, and adenocarcinomas (Table 2).[24,26]

Despite much work on other types of tumors, there have been very few reports of *EGFR* mutations outside of lung carcinomas. The reported rates of *EGFR* mutation in lung cancers are variable and are obviously highly dependent on the population being tested. The *EGFR* mutations are clustered in the region of exons 18 to 21, which is the area that encodes the ATP-binding pocket of the tyrosine kinase domain of *EGFR*. The most common mutations involve exons 19 and 21, which account for more than 95% of mutations. Exon 19 mutations are deletion mutations, usually involving between 10 and 20 base pairs of the exon 19 sequence, beginning around codon 746; occasional exon 19 point mutations are also seen (codon 719). Exon 21 mutations are almost always point mutations, involving codon 858 with a T-to-G transversion in most cases.

The relationship between somatic *EGFR* sequence mutations and response to anti-EGFR therapies has been controversial. Initial reports were promising, with demonstrated tumor shrinkage and benefits in surrogate endpoints.[23,25] It appeared that mutations in the *EGFR* gene were more frequent in patients who responded to anti-EGFR therapies. However, importantly, all of these reports also acknowledge that some patients without identifiable mutations responded to anti-EGFR therapy. More recent studies have failed to demonstrate long-term benefit in survival advantages with treatment with anti-EGFR therapies.[27,28] Therefore, testing for the point mutations and deletion mutations in the *EGFR* gene has not become standard practice at this time.

CONCLUSION

This overview provides several examples of molecular tests in different stages of clinical applicability. The life cycle of new diagnostic assays is complex. Some assays quickly and appropriately become the standard of care. Others are brought into clinical practice slowly, as needs evolve and develop. Still others show initial promising results but are abandoned when they fail to provide value in practice or when clinical management changes over time. The assays discussed provide examples that fit into each of these categories.

Molecular testing in anatomic pathology will almost certainly become critical for providing optimal patient care during the next 5 to 10 years. As more assays are developed that provide valuable diagnostic, prognostic, and therapeutic information for patient management, anatomic pathologists will need to be familiar with the literature to order appropriate ancillary studies. Advanced molecular assays are best interpreted in an integrated fashion, with basic hematoxylin-eosin morphology and immunohistochemical findings remaining an essential component of the overall diagnosis.

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